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Short communication

In vitro eye irritation testing using the open source reconstructed hemicornea – a ring trial

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Summary

Aim of the present ring trial was to prove whether two new methodological approaches for the *in vitro* classification of eye irritating chemicals can be reliably transferred from the developers' laboratories to other sites. Both test methods are based on the well-established open source reconstructed 3D hemicornea models. In the first approach, the initial depth of injury in the hemicornea model after chemical treatment is derived from the quantitative analysis of histological sections. In the second approach tissue viability, as a measure for corneal damage after chemical treatment, is analyzed separately for epithelium and stroma of the hemicornea model. The 3 independent laboratories which participated in the ring trial produced their own hemicornea models according to the test producer's instructions, thus supporting the open source concept. A total of 9 chemicals with different physico-chemical and eye-irritating properties were tested to assess the between-laboratory reproducibility (BLR), the predictive performance as well as possible limitations of the test systems.

The BLR was 62.5% for the first and 100% for the second method. Both methods enabled to discriminate cat 1 chemicals from all non-cat 1 substances, which qualifies them to be used in a top-down approach. However, the selectivity between no cat and cat 2 chemicals still needs optimization.

Keywords: *In vitro* eye irritation testing, Open source 3D hemicornea equivalent, depth of injury, ring trial, test performance

1 Introduction

In order to replace the Draize Eye Irritation Test (OECD TG 405) different approaches have been pursued to develop animal-free alternative test methods. Currently 5 *in vitro* methods are available that have undergone formal validation and eventually gained regulatory acceptance (OECD TG 437, 438, 460, 491, 492). Four of them, the Isolated chicken eye test (ICE), the Bovine corneal opacity and permeability assay (BCOP), the Short time exposure test (STE) and the Reconstructed human cornea-like epithelium test *(*RhCE*)* can be used to identify chemicals which do not require a GHS classification (no category) or which induce serious eye irritation (GHS category 1). In contrast, the fluorescein leakage assay is only accepted for the classification of serious eye damage. Thus, classification of a chemical as a GHS category 2 substance is based on the exclusion principle: if a chemical cannot be identified as a category 1 substance or as non-irritant for the eye, it is assigned to GHS category 2. In order to overcome this limitation in predictivity two different test methods, both based on a bioartificially produced 3D human corneal equivalent (hemicornea), have been developed (Zorn-Kruppa et al., 2014, Bartok et al., 2015). The hemicornea model consists of a differentiated epithelium on top of a collagen gel populated with stromal keratocytes (Zorn-Kruppa et al., 2004, 2005, Engelke et al., 2013). With this complex tissue architecture, mimicking epithelium and stroma of the human cornea, the hemicornea comprises essential properties which had been recognized by an expert group to be a prerequisite for any test method to predict all GHS categories (Scott et al., 2010). For the first method the "depth of injury" (DOI) concept, based on extensive analyses performed by Jester and colleagues on isolated rabbit eyes (Jester et al., 2010), has been adapted. The depth of tissue damage after chemical exposure is determined on histological sections of the treated hemicornea models and is a measure for the eye-irritating potential of the test item. Assay performance, reliability and predictivity of both methods have been demonstrated in the developers' laboratories with sets of reference chemicals representing all GHS categories and different physicochemical properties (Zorn-Kruppa et al., 2014; Bartok et al., 2015; Tandon et al., 2015).

In the second method (collagen cell carrier –"CCC" approach) epithelium and stroma of the hemicornea become physically separated by an artificial collagen membrane inserted at the interface between both tissues before seeding of the

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epithelial cells. After chemical exposure of these modified models, the epithelium can be easily stripped off the stroma, and cell viability can be analyzed separately for each tissue. This procedure takes into account the observation that certain chemicals are known to damage epithelium and stroma differently (Jester et al., 1998a,b, 2001, 2006, 2010, Maurer et al., 2001, 2002).

The aim of the present ring trial is to prove that the protocols for both tests can be reliably transferred from the developers' laboratories to other sites to prove both the between- laboratory reproducibility (BLR) and the predictive performance. Both methods had been established in order to predict the eye-irritation potential of chemicals for all 3 GHS categories within one test.

2 Materials and methods

The protocols for the production of the hemicornea models and the performance of the eye irritation test have been previously published by Zorn-Kruppa et al., 2014, and Bartok et al., 2015. More details, including the list of test items used in this ring trial, can be found in the supplementary file at https://doi.org/10.14573/altex.1610311s.

Three laboratories participated in the ring trial: Henkel AG & Co. KGaA (Lab 1), University Medical Center Hamburg-Eppendorf (Lab 2, developers lab for DOI approach), and Jacobs University Bremen (Lab 3, developers lab for CCC approach). The participating laboratories produced their own hemicornea models for both methodological approaches. The test chemicals for all participants were taken from the same batch and distributed blinded. All chemicals were tested in 3 independent runs for each method in each laboratory.

3 Results and discussion

DOI Method

Nine chemicals were tested initially. However, lactic acid could not be classified due to tissue disintegration after substance application, concordantly observed in all 3 laboratories. From the remaining 8 chemicals, 5 were tested concordantly in all laboratories, leading to a between-laboratory reproducibility of 62.5%. Three chemicals were classified discordantly. The within-laboratory reproducibility was 62.5%, 87.5% and 62.5% for laboratories 1, 2, and 3, respectively (data not shown in this paper).

From the 3 chemicals, which are classified as non-irritants in the Draize test only dodecane was predicted correctly in all 3 test laboratories (Tab. 1). Topical exposure to n-butyl acetate and iso-propyl bromide resulted in 23-41% mean damage of the corneal tissue. Hence, they were classified as GHS category 2 chemicals (false positives). All 3 chemicals were classified concordantly in the laboratories.

Tab. 1: Initial Depth of Injury (DOI) in hemicornea models after topical exposure to chemicals of different eye-irritation potentials

Chemical	***in vivo*** **GHS cat.**	**Lab 1** DOI [%]	*in vitro* class	**Lab 2** DOI [%]	*in vitro* class	**Lab 3** DOI [%]	*in vitro* class	**BLR**
Methyl pentynol	Cat 1	94.02 ± 5.87	Cat. 1	95.97 ± 2.84	Cat. 1	89.88 ± 3.19	Cat. 2	dis
1,2,4-Triazole Na salt	Cat 1	99.20 ± 0.70	Cat. 1	99.98 ± 0.02	Cat. 1	99.69 ± 0.12	Cat. 1	con
Lactic acid (100%)	Cat 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	con
4-Carboxybenzaldehyde	Cat 2A	3.48 ± 2.28	No Cat.	5.16 ± 2.23	Cat. 2	1.98 ± 0.67	No. Cat.	dis
n-Hexanol	Cat 2A	78.02 ± 1.72	Cat. 2	75.83 ± 11.11	Cat. 2	90.30 ± 5.26	Cat. 1	dis
Ethyl-2-methyl acetoacetate	Cat 2B	8.94 ± 4.41	Cat. 2	26.13 ± 5.88	Cat. 2	9.13 ± 11.03	Cat. 2	con
Dodecane	No Cat	1.40 ± 0.28	No Cat.	0.14 ± 0.09	No Cat.	1.06 ± 0.08	No Cat.	con
n-Butyl acetate	No Cat	23.72 ± 9.48	Cat. 2	36.74 ± 1.54	Cat. 2	25.90 ± 2.52	Cat. 2	con
iso-Propyl bromide	No Cat	22.58 ± 6.81	Cat. 2	30.29 ± 1.77	Cat. 2	40.61 ± 2.96	Cat. 2	con

Mean DOI values +/- standard deviation for 3 independent test runs of 3 hemicornea tissues each are shown for all laboratories. For every laboratory, the resulting GHS classification according to the prediction model is indicated. No Cat. – not classified (not irritation to the eye); Cat. 2 – moderately irritating to the eye; Cat. 1 – seriously irritating to the eye; n.d. – not determined. The values highlighted in grey indicate the false predictions. The BLR of test results for every chemical is mentioned (dis – discordant results; con - concordant results) as well as the in vitro classification based on the majority of results achieved in the 3 laboratories. Dodecane was tested only twice at Lab 1.

The results for the moderately eye-irritating chemicals (GHS category 2) were quite heterogeneous with mean DOI values between 1 and 91%. 4-carboxy benzaldehyde was clearly misclassified as non-irritant in two laboratories. In the 3rd laboratory the mean DOI of 5.16% was only slightly above the cut-off value of 5% which separates non-classified from category 2 chemicals. 4-carboxy benzaldehyde is a solid; the misclassification (false negative) probably resulted from the low solubility on the tissue surface. In contrast to liquid substances, most of the applied solid matter was not in cell contact and did not penetrate the tissue. Ethyl-2-methyl acetoacetate was concordantly predicted correctly in all laboratories. The results for n-hexanol were discordant due to the prediction as category 1 chemical in laboratory 3. However, the respective mean DOI value is only marginally above the cut-off value of 90% which separates category 1 and 2. An in-depth analysis of the data generated in 3 independent runs in Lab 3 revealed that 2 out of 3 runs resulted in DOI values above and 1 value below the 90% cut-off value. Thus, the individual results are discordant, too, with the median characterizing n-hexanol as cat. 1 chemical.

Two out of 3 chemicals classified as GHS category 1 could be analyzed properly in this approach. After topical application of lactic acid (100%) the collagen gel dissolved which led to a complete hemicornea disintegration. Thus, the DOI could not be determined, because it depends on an acceptable tissue preservation. The 2 other chemicals both led to massive damages of the hemicornea tissues. 1,2,4-triazole Na salt was concordantly predicted correctly as being corrosive to the eye (GHS category 1). The test of methyl pentynol led to discordant results, because the DOI mean was below the 90% cut-off value in laboratory 3. Only two of 3 independent runs resulted in values >90% DOI.

The results of this part of the ring trial revealed a tendency which was already observed during the developmental phase and protocol transfer of this method (Zorn-Kruppa et al., 2014). According to these observations the DOI data for the category 1 chemicals all fell into a quite narrow range of values above the 90% cut-off threshold which unambiguously identified them as corrosive to the eye. In contrast, the DOI values for the category 2 chemicals varied over a broad range, a fact that is also reflected in the prediction model.

This study also reveals limits with regard to the applicability of chemicals, which disintegrate the tissue structure. pH-extreme chemicals like acid and alkaline solutions are apparently not compatible with the hemicornea model which is based on a collagen gel. Upon exposure with an acid the collagen gel liquefies and hence, cannot be fixed and stained for further analysis. A similar effect was observed with aqueous SDS solutions, which also disintegrate the tissue structure without the chance for reliable DOI assessment (data not shown in this paper). Thus, it is inevitable to check whether the substances to be tested for their eye-irritating potential fall into the respective applicability domain of the method. On the other hand, a clear advantage of the DOI method is its potential to gain more information about the mode of action of a given chemical within the corneal tissue since it is based on histological methods. Furthermore, it is noteworthy that in lab 2 which is the developer's lab for the DOI approach this method achieved the best results of the entire study regarding correct classification.

Tab 2: Relative viability data generated with the CCC method (separated epithelium and stroma after topical exposure of hemicornea models with chemicals of different eye-irritation potentials)

Chemical	*In vivo* GHS category	Viability [%] ± SD						*In vitro* classification (identical in all labs)
		Epithelium			Stroma			
		Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	
Methyl pentynol	Cat 1	1.09 ± 0.26	1.94 ± 0.27	1.92 ± 0.68	17.78 ± 10.19	18.17 ± 2.22	16.36 ± 4.51	Cat. 1
1,2,4-Triazole Na salt	Cat 1	2.67 ± 0.17	3.68 ± 0.73	3.82 ± 0.24	8.23 ± 1.24	19.01 ± 2.16	26.12 ± 12.76	Cat. 1
Lactic acid (100%)	Cat 1	1.81 ± 0.53	2.65 ± 0.30	3.04 ± 1.54	1.26 ± 0.86	0.50 ± 0.15	2.23 ± 0.76	Cat. 1
4-Carboxybenzaldehyde	Cat 2A	55.07 ± 26.22	59.52 ± 6.26	69.51 ± 14.61	76.41 ± 26.22	99.76 ± 9.34	105.31 ± 26.52	No Cat.
n-Hexanol	Cat 2A	1.38 ± 0.71	2.28 ± 0.60	2.84 ± 0.27	38.94 ± 13.76	35.72 ± 11.82	67.83 ± 3.57	Cat. 2
Ethyl-2-methyl acetoacetate	Cat 2B	6.48 ± 6.76	2.24 ± 0.58	5.20 ± 3.22	68.42 ± 29.25	78.14 ± 9.47	83.35 ± 8.57	Cat. 2
Dodecane	No Cat	79.59 ± 31.66	75.13 ± 6.03	107.10 ± 26.63	83.72 ± 25.66	97.49 ± 5.20	96.60 ± 1576	No Cat.
n-Butyl acetate	No Cat	1.76 ± 1.53	2.08 ± 0.84	2.05 ± 0.29	50.02 ± 20.07	68.05 ± 21.67	81.10 ± 17.47	Cat. 2
iso-Propyl bromide	No Cat	4.11 ± 3.21	2.09 ± 0.40	2.83 ± 0.56	35.90 ± 2.44	36.71 ± 3.43	36.81 ± 5.81	Cat. 2

The columns show the mean values of relative tissue viability for three independent experiments +/- standard deviations. Each test run was performed with 3 hemicornea models. In the right column, the in vitro, based on the majority of classification from the 3 labs, is indicated: No Cat. – not classified (not irritation to the eye); Cat. 2 – moderately irritating to the eye; Cat. 1 – seriously irritating to the eye. Relative tissue viability is calculated related to the respective negative control (100% tissue viability). The fields highlighted in grey indicate false negative and false positive results, respectively, as compared to the in vivo classification, which were common to all labs. Ethyl-2-methyl acetoacetate was tested only twice at JU Bremen.

CCC method

All 9 chemicals were concordantly classified in all participating laboratories, which corresponds with a between laboratory reproducibility of 100%.

All chemicals classified as GHS category 1, based on the Draize test, were predicted correctly in all 3 laboratories (table 2). Relative viabilities were clearly below the cut-off thresholds of 15% and 35% for the epithelium and the stromal compartment, respectively. In contrast to the DOI method, the eye- irritating potential of lactic acid could be determined using the CCC approach, because the epithelial part could be removed from the stroma and transferred to another well while the stroma remained in the insert. Cell viability could be determined in both compartments. Two out of 3 GHS category 2 chemicals were predicted correctly, with mean epithelial viability values below 7%. Ethyl-2-methyl acetoacetate was classified as category 2 with relatively high stromal tissue viabilities. This chemical is classified as GHS category 2B and the *in vitro* result reflects its lower *in vivo* eye irritation potential. 4-carboxy benzaldehyde was misclassified as a non-irritant in all test laboratories, based on both high epithelial and stromal viability. Poor solubility and hence poor bioavailability are considered to be responsible for this result in the CCC test, too.

From the 3 non-irritating chemicals, only dodecane was predicted correctly in all laboratories. In contrast, both n-butyl acetate and iso-propyl bromide were classified as GHS category 2 chemicals. Both chemicals resulted in very low epithelial viabilities below 5%, and for iso-propyl bromide even the mean stromal viabilities of about 36-37% were very close to the 35% cut-off value which distinguishes categories 1 and 2.

The results of the eye irritation test conducted with the CCC method correspond with those generated with the DOI approach. GHS category 1 chemicals were all predicted correctly, characterized by low epithelial and stromal relative tissue viability. The selectivity between cat 1 and cat 2 chemicals was good, and no cat 2 or non-irritant chemical was overpredicted as cat 1. These observations confirm the outcome from previous studies conducted on hemicornea tissues. In contrast, the discriminatory power between cat 2 and no cat chemicals is still too low.

4 Conclusions

Irrespective of whether the relative viability after topical treatment with chemicals was determined in an MTT assay of the whole tissue (Engelke et al., 2013), or whether sets of chemicals different from those used in the current study were assessed with the CCC and DOI methods, respectively (Zorn-Kruppa et al., 2014; Tandon et al., 2015; Bartok et al., 2015), the GHS category 1 chemicals were always clearly separated from the other chemicals. Thus, both hemicornea-based test methods presented in this paper are suited to be used in a top-down approach to single out category 1 chemicals with high reliability, a condition already previously requested by an EURL-ECVAM expert team in 2005 (Scott et al., 2010). The 2 non-irritating chemicals which had been classified as false positives as well as one category 2 chemical misclassified as a non-irritant were identical in both methods.

In conclusion, the ring trial presented here proved that both hemicornea-based in vitro methods to assess the eye-irritation potential of chemicals can be successfully transferred to other laboratories. However, a lower between laboratory reproducibility was observed for the DOI method. This difference could be attributed to the high complexity of the DOI method, requiring good technical skills, and to some borderline values in the close vicinity of the respective cut-off values of the prediction model (e.g. for cat 1 chemical methyl pentynol). The predictive capacities were comparable for both assays and thus confirmed results from previous studies conducted with the hemicornea (Engelke et al., 2013; Bartok et al, 2015; Tandon et al., 2015; Zorn-Kruppa et al., 2014), whereas the selectivity of both assays must be optimized.

Both methods presented in this paper repeatedly revealed their strength to clearly distinguish cat 1 chemicals from all non-cat 1 substances. Thus, they could be used in a top-down approach to identify those chemicals leading to severe eye damage in the first instance (Scott et al., 2010). In addition, these methods are further examples for the open source concept, meaning that all protocols underlying tissue production and assay performance have been made publicly available without any legal and intellectual property restrictions for the indicated purpose, given that the predefined quality criteria are met.

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Conflict of interest

The authors declare that they have no conflict of interests.

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